SUPPRESSION OF DEFECTIVE MOTOR PATTERNS IN PARKINSONIAN C. ELEGANS

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1. ABSTRACT

Parkinson's disease (PD) is a progressive neurodegenerative disorder that is caused partly by the loss of dopamine producing neurons. Dopamine is a conserved neuromodulator that aids in the transition between different motor patterns such as swimming, walking, or running. This can be observed across many species, including mice, flies, and nematodes. The *cat-2* mutant strain of the nematode *C. elegans* has a deletion in the gene encoding tyrosine hydroxylase, an enzyme required to synthesize dopamine. Our lab recently demonstrated that the *cat-2* mutant shares aspects of PD patient dysfunction through its inability to transition normally between the "swimming" and "crawling" patterns of motion. Currently, PD treatments focus on boosting residual dopamine signaling and are not available to maintain motor function once dopamine neurons completely degenerate. To search for ways to overcome motor dysfunction in the absence of dopamine, we performed a forward genetic screen to identify mutations that suppress poor swim-to-crawl motor transition in *cat-2* mutant. We found several suppressor mutants that improve motor function. Further characterization can identify molecular pathways that can be altered to improve motor function in the absence of dopamine. This information could provide insight into repair of dopamine-deficient neural circuitry in higher level animals and possible approaches to help late stage PD patients.

2. INTRODUCTION

Many organisms exhibit distinct forms of motion, known as gaits, and switch between different gaits in response to various stimuli. For instance, horses display three distinct gaits through walking, trotting, and galloping (Alexander, 2003); similarly, humans switch between gaits when they transition from walking to running. The molecule dopamine has been shown to modulate such gait transition across a wide range of taxa. The unique ability of dopamine to regulate locomotion may stem from the large set of dopamine receptors that exist across species (Vidal-Gadea & Pierce-Shimomura, 2012). These receptors are divided into numerous subtypes. D1-like receptors activate adenylyl cyclase upon dopamine stimulation, resulting in increased production of cyclic AMP (cAMP); on the other hand, D2-like receptors inhibit adenylyl cyclase and decrease levels of cAMP (Neves, 2002). The opposing nature of these receptor subtypes allow dopamine signaling to finely regulate molecular pathways such as motor pattern transition.

The connection between dopamine signaling and motor function can be observed through the development of Parkinson's disease (PD), the second most common neurodegenerative disorder in humans (Lebouvier et. al., 2008). Common physical symptoms of Parkinson's include rigidity, shaking, freezing, and inability to begin voluntary movements. Physiologically, Parkinson's disease is characterized by the death of

dopaminergic neurons in the substantia nigra, along with other heterogenic populations of neurons (Lang & Lozano, 1998). The substantia nigra is a region of the midbrain that is responsible for voluntary movements and communicating signals from the basal ganglia to other brain structures (Guatteo et. al., 2009). The nigrostriatal pathway between the substantia nigra and the dorsal striatum regulates motor function; when nigrostriatal dopamine binds to D1 and D2 dopamine receptors in the striata, muscle contractions occur and muscle tones change (Guatteo et. al., 2009). When PD has reached advanced stages and symptoms manifest, approximately 50% of neurons in the nigrostriatal pathway have degenerated. Therefore, the death of dopaminergic neurons in this pathway has severe consequences for the motor activity of late stage PD patients.

Currently, there are no available treatments that can halt the progression of the disease; instead, many existing treatments aim to alleviate symptoms. The most commonly used treatment for PD is L-Dopa, a precursor to dopamine. However, treatment with L-Dopa is unable to resolve severe motor disabilities and continued usage of the medication can exacerbate tremors and dyskinesia (Nadjar, Gerfen, & Bezard, 2009). In some cases of advanced PD, deep brain stimulation is used to activate remaining dopaminergic neurons. However, this procedure carries the risk of severe surgical and neuropsychiatric side effects (Pienaar et. al., 2010). Thus, there is a need for treatment that resolves motor deficits in late-stage PD.

To examine suppression of Parkinsonian motor function in the absence of dopamine, we utilized the genetically tractable nematode, *Caenorhabditis elegans.* These roundworms are advantageous as animal models because they have a short life span, reaching adulthood within 4 days, are affordable, and produce large amounts of progeny. Furthermore, previous experiments have demonstrated that *C. elegans* can display distinct motor patterns (Vidal-Gadea et al; 2011). During the crawling pattern, the nematode propagates a slow, deep bend down the length of the body, while during the swimming pattern, the bends are shallow and rapid. Dopamine is necessary and sufficient to modulate the swim-to-crawl gait transition in *C. elegans.* The *cat-2* strain of *C. elegans* has a 211 bp deletion in the gene for tyrosine hydroxylase, the enzyme that produces L-dopa in the dopamine synthesis pathway; thus, this strain lacks the ability to produce dopamine (Bettinger et. al., 2004). Disruption of dopamine signaling in the *cat-2* strain results in defective gait switching, analogous to the motor deficits experienced by late stage PD patients (Vidal-Gadea et. al., 2011). Other types of PD models rely on the introduction and overexpression of the gene α-synuclein, which leads to Lewy body formation and subsequent dopaminergic neuron degeneration (Dauer & Przedborski, 2003). While these models are useful for studying ways to prevent neuron death, this project seeks to retain dopamine-dependent nervous system function; thus, α-synuclein based models are not utilized in these experiments.

Here, a forward genetic screen was used to introduce random mutations into the *cat-2* model to uncover rare mutants that suppress the Parkinsonian swim-to-crawl motor deficit. From this screen, we isolated four viable suppressor mutants that were able to transition between gaits equally or more efficiently than the wildtype strain. Through the following experiments, we aimed to further characterize these suppressor mutants by analyzing dopamine-mediated behaviors.

3. METHODS

3.1: C. elegans husbandry and maintenance

C. elegans were grown on nematode growth media (NGM) agar plates seeded with OP50 bacteria at 20 degrees Celsius as previously described (Brenner 1974). The N2 strain was used as the wild-type. For a list of the strains and allele designations used in this experiment, see Figure 1.

Strain	**Allele Designation**	**Background/Description**
N2	-	Wild-type
cat-2 (JPS651)	-	PD model
JPS871	*vx26*	Suppressor mutant with JPS651 background
JPS872	*vx27*	Suppressor mutant with JPS651 background
JPS873	*vx28*	Suppressor mutant with JPS651 background
JPS896	*vx29*	Suppressor mutant with JPS651 background

Figure 1: *C. elegans* Strain Characteristics. The N2 and *cat-2* strains were obtained from the Caenorhabditis Genetics Center. Suppressor mutants from the forward genetic screen were assigned strain names using the designated lab prefix (JPS) and were also assigned unique allele names.

3.2: Forward genetic screen

The *cat-2* mutant strain, which carries the 211 bp deletion for the tyrosine hydroxylase gene, was used for forward mutagenesis because the nature of the deletion makes the uncovering of revertant mutants from the suppression screen unlikely. A population of the *cat-2* strain at the L4 stage was incubated in *N*-methyl-*N*-nitrosourea (ENU) for four hours. After incubation, the worms were rinsed in NGM and allowed to recover for three hours on a plate seeded with OP50. 100 of these mutagenized *cat-2* individuals, referred to as the P0 generation, were picked and distributed on 10 different plates. After the P0 individuals reached adulthood, they laid eggs for 12 hours (F1 generation) on each of the 10 plates. The F1 worms reached adulthood and laid eggs, giving rise to the F2 generation. Lastly, as the F2 generation reached adulthood, they were screened as a population for swim to crawl transition efficiency through a radial dispersion assay.

3.3: Radial Dispersion Assays

The swim to crawl efficiency of a strain on a population level was assessed by modifying previously used methods of evaluating locomotion (Vidal-Gadea et. al., 2011). For this assay, unblemished 6 cm agar plates were selected and prepared by placing a solution of diacetyl along the circumference of the plate. Four evenly spaced 2 µL droplets of diacetyl were pipetted onto the plate's perimeter and a watercolor brush was used to spread the diacetyl solution around the circumference. The placement of the diacetyl motivates the worms to crawl towards the outer edges of the agar.

Populations of worms were tested on either the first or second day of adulthood. Approximately 50-100 age matched adult worms from a population were picked and transferred from agar plates to an Eppendorf tube containing 1.5 mL of NGM. The worms were rinsed twice with NGM to remove any residual OP50 that was transferred along with the worms. Then, a Drummond pipette fitted with a 25 µL capillary tube was used to transfer the worms from the Eppendorf tube to the prepared agar plate with diacetyl by ejecting a small (5-10 µL) puddle of worms suspended in buffer onto the center of the plate.

The worms were then transferred to a recording chamber that used darkfield style illumination. A USB microscope was used to record the worms transitioning from swimming in the puddle to crawling on the plate. The time lapse recording starts just prior to the worms beginning to emerge from the puddle and continues for two minutes, capturing the video at a rate of 1 frame per 2 seconds.

The resulting 2 minute time lapse recording was processed and analyzed with ImageJ processing software. The images of the plate were divided into four concentric zones, as shown in Figure 2. Manual counts of the number of worms in each zone at the 0, 20, 40, 60, 80, 100, and 120 second intervals were recorded for each population.

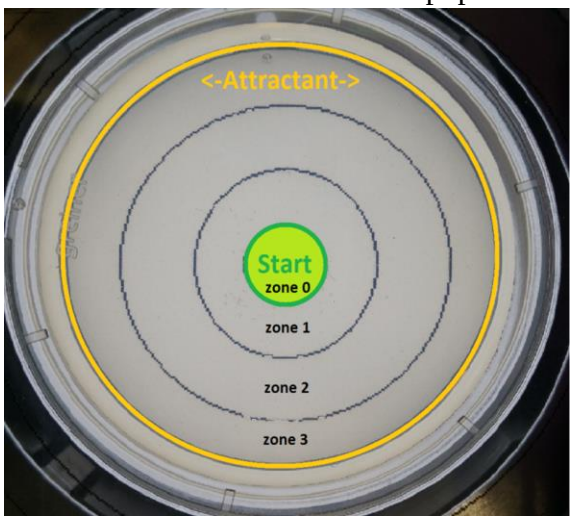

Figure 2: Radial Dispersion Setup and Analysis. An ImageJ macro was created that divided each image of the plate into four concentric zones, as shown above. For each of the specified time intervals, manual counts of the number of worms were recorded for subsequent analysis.

3.4: Treatment with 6-hydroxydopamine

To observe the behavior of worm populations under induced dopamine neuron degeneration, previous methods utilizing 6-hydroxydopamine (6-OHDA) were modified (Nass, Hall, Miller, & Blakely, 2002). As 6-hydroxydopmaine oxidizes quickly and easily, the antioxidant ascorbic acid was added to each of the solutions.

In preparation for 6-OHDA treatment, an age matched population of worms at the L4 stage was transferred from an agar plate to an Eppendorf tube and was rinsed three times with dH_2O to remove residual OP50 bacteria. 250 µL of the L4 worms suspended in dH_2O was then added to a 250 µL solution of 60 mM 6-OHDA/80 mM ascorbic acid. The resulting

tubes was mixed, resulting in the worms suspended in 500 µL of 30 mM 6-OHDA/40 mM ascorbic acid solution. The tube was then gently mixed for 30 minutes at room temperature. After the 30 minutes, the worms were rinsed with dH_2O and transferred to a seeded agar plate for recovery. After 24 hours of recovery, the worm population was tested for swim-to-crawl efficiency using the radial dispersion assay.

3.5: Exogenous dopamine treatment

The behavior of the suppressor mutant strains when treated with exogenous dopamine was analyzed by adding slight modifications to the radial dispersion procedure. Worms that have reached the first or second day of adulthood were transferred from an agar plate to an Eppendorf tube, and were then rinsed with NGM twice. 100 µL of the rinsed worms suspended in the NGM buffer was then mixed with 100 µL of a 50 mM dopamine solution, leaving the worms immersed in a 25 mM dopamine solution. After the worms soaked in the exogenous dopamine for 5 minutes, they were rinsed twice with NGM. A Drummond pipette was used to pipette the worms in a 5-10 µL puddle at the center of a plate lined with diacetyl, as previously described. The radial dispersion assay is then performed.

3.6: Outcrossing

To prepare the suppressor mutant strains for whole genome sequencing, they were outcrossed against their parent strain *cat-2* to remove any background mutations not related to motor deficit suppression.

To backcross the suppressor mutants, hermaphrodites from the suppressor mutants were crossed against males from the *cat-2* strain. These males were generated by incubating *cat-2* hermaphrodites in the L4 stage for 3 hours at 30 degrees Celsius. After this heat shock procedure, males were isolated from the F1 generation. 6 *cat-2* male worms and 2 hermaphrodites from the suppressor mutants were placed together on a plate and allowed to mate for 24 hours. After this time passed, the hermaphrodites were picked onto a separate plate. Once the F1 progeny of these mated hermaphrodites reached the L4 stage, 10 random worms were picked and placed onto 10 individual plates (1 worm/plate). Then, when the F2 progeny of each of these 10 worms reached adulthood, the adult worms were evaluated in a radial dispersion assay. The worms that still displayed the most efficient swim-to-crawl transitions and suppression of the *cat-2* motor deficit were isolated to be used for further backcrossing.

In order to maximize removal of background mutations, 6 total backcrosses were performed for every mutant strain.

4. RESULTS

4.1: Absence of dopamine impairs swim-to-crawl transition

To examine gait transition in *C. elegans* in the absence of dopamine, we conducted radial dispersion assays on the wildtype strain and the *cat-2* PD model. While the 120 second duration of the assay was a sufficient time interval for approximately half of the worms to reach the outer two zones, the majority of *cat-2* worms remained in the inner zones at this time point. By the end of the assay, a significantly larger proportion of wildtype worms

(15.01% ± 13.2) reached the outermost zone, zone 3, when compared to the *cat-2* worms (2.1% ± 4.4).

Qualitative observations of the time lapse recordings showed that while wildtype worms reach the outer zones by moving at a steady pace, *cat-2* worms tend to freeze and reverse in the inner zones of the plate. As *cat-2* worms emerge from the puddle and attempt to initiate crawling behavior, they exhibit unusual flaccid posture and are not able to propagate bends along the length of their body as quickly as wild type worms.

Therefore, the results of these experiments confirm that *cat-2* displays the expected swim-to-crawl motor deficit and that the radial dispersion assay is sufficient to capture motor activity.

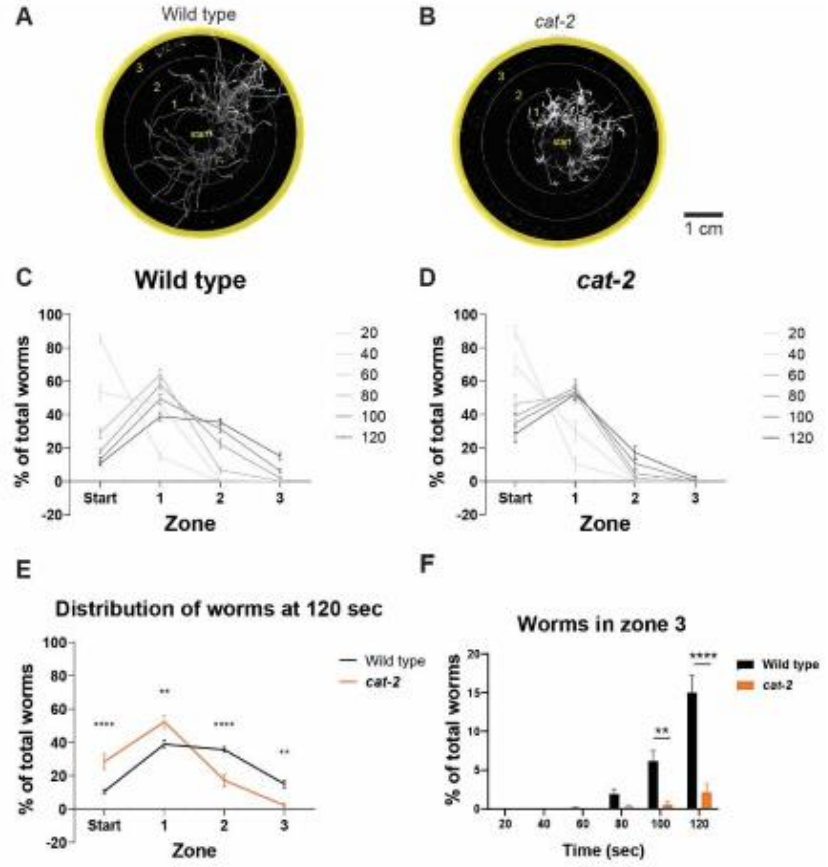

Figure 3: Radial dispersion of wildtype and *cat-2* (PD model) strains. Radial dispersion assays were set up as shown in Figure . (A) an illustration of

4.2: Forward genetic screen uncovers mutations that suppress motor deficit

After mutagenesis of *cat-2* with ENU, progeny were screened for regain of swim-to-crawl transition. Four suppressor mutants that successfully suppressed the Parkinsonian motor phenotype were isolated and assigned strain and allele names as documented in Figure 1. Multiple radial dispersion assays were performed on each of these suppressor strains to evaluate their abilities to switch gaits. As shown in Figure 4 G and H, each suppressor mutant population had a higher percentage of worms in outer zones when compared to *cat-2*. Of

particular interest, the *vx28* strain showed a significantly larger proportion of worms reaching the outermost zone even when compared to the wildtype strain.

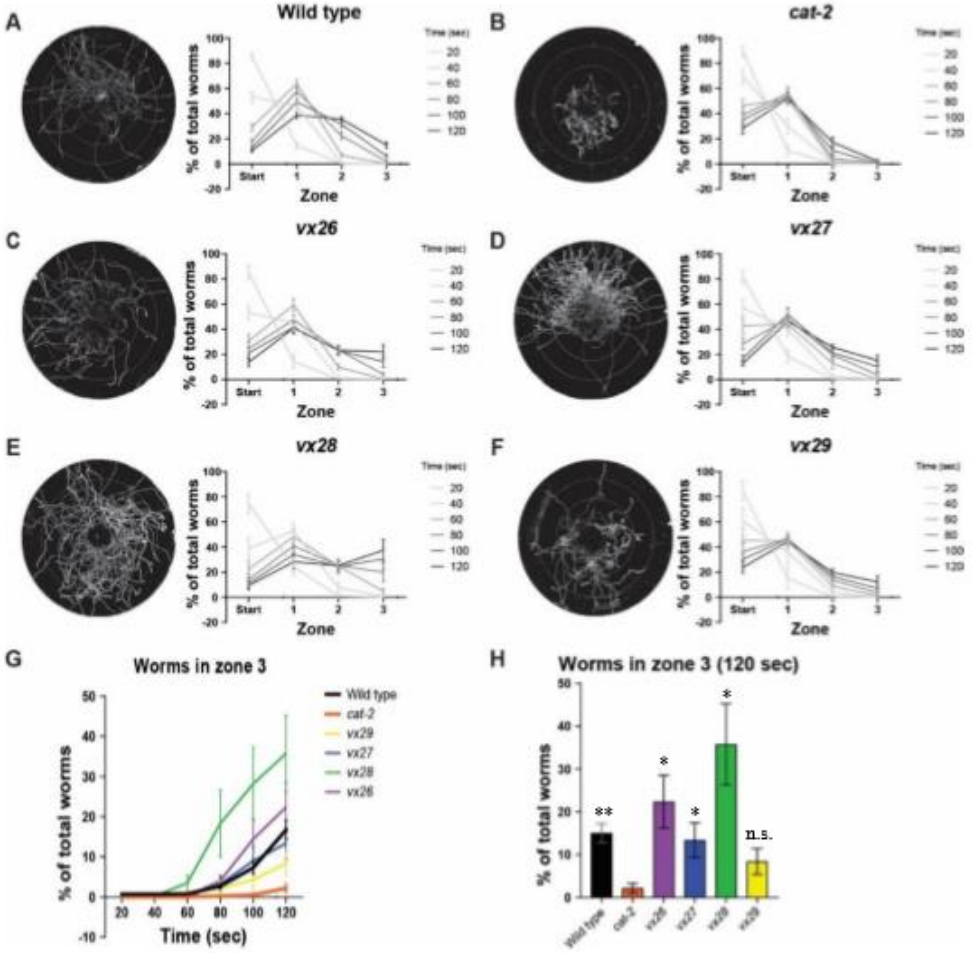

Figure 4: Radial dispersion of suppressor mutants from forward genetic screen.

4.3: Improved motor function of suppressor mutants not explained by hyperactivity

One potential explanation for the increased efficiency of gait transition in suppressor mutants may be locomotor hyperactivity; the worms may simply be crawling faster instead of regaining the ability to transition between motor patterns. To test this hypothesis, the worms were tested in a radial dispersion assay that did not require a swim to crawl transition; instead, the worms were picked to the center of the plate without being placed in a puddle, and crawled for 2 minutes. The results of this "dry" radial dispersion assay show no significant difference between the proportion of worms able to reach the outermost zone at any of the time points measured.

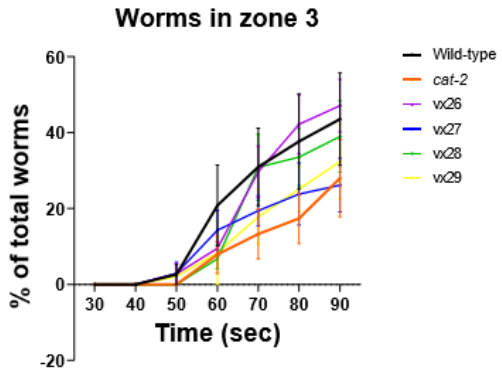

Figure 5: Dry radial dispersion of suppressor mutants.

4.4: Induced dopaminergic neuron degeneration did not significantly impair mutant motor function

When treated with 6-OHDA, the wildtype strain performed significantly worse on the radial dispersion assay, at a level comparable to *cat-2*. There were no significant differences between untreated worms and worms treated with 6-OHDA for any of the four mutant strains. However, it is important to note that *vx26* trends towards increased performance following 6-OHDA treatment, while *vx27* trends towards decreased performance. The nonsignificant difference between untreated and treated worms may stem from the small sample size used in this assay. Additional trials are needed to validate the observed results from 6-OHDA exposure.

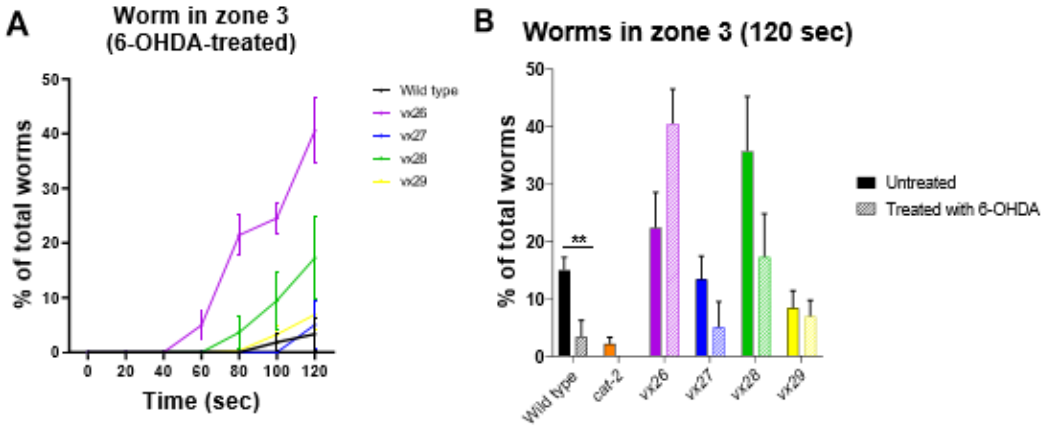

Figure 6: Motor function of suppressor mutants following 6-OHDA treatment. Radial dispersion assays were performed on populations of worms exposed to 6-OHDA. (A) illustrates the proportion of worms that reached the outermost region, zone 3, at each time interval in the 2 minute radial dispersion assay. (B) shows the proportion of worms that reached zone 3 at the 2 minute time point for both untreated worms and worms treated with 6-OHDA. Wild type (N = 6, $p < 0.0079$), *cat-2* untreated (N = 15), vx26 (N = 4, $p = 0.0526$), vx27 (N = 5, $p = 0.1214$), vx28 (N = 5, $p = 0.1062$) and vx29 (N = 5, $p = 0.3511$).

4.5: Exogenous dopamine treatment shows variable effects on mutant strains

As the suppressor mutant strains are able to successfully transition between motor patterns in the absence of dopamine, we explored how the presence of dopamine could affect their motor activity. As expected, an increased proportion of *cat-2* worms were able to reach zone 3 following exogenous dopamine treatment. Two strains, *vx26* and *vx28*, showed a decreased ability to transition between swimming and crawling after exogenous dopamine treatment; moreover, the decrease in the percentage of worms able to reach the outermost zone was significant in *vx28*. *vx27* showed a slight increase in gait transition efficiency after exogenous dopamine treatment, but the change was nonsignificant. Lastly, the *vx29* did not show any significant change in motor function after exogenous dopamine treatment.

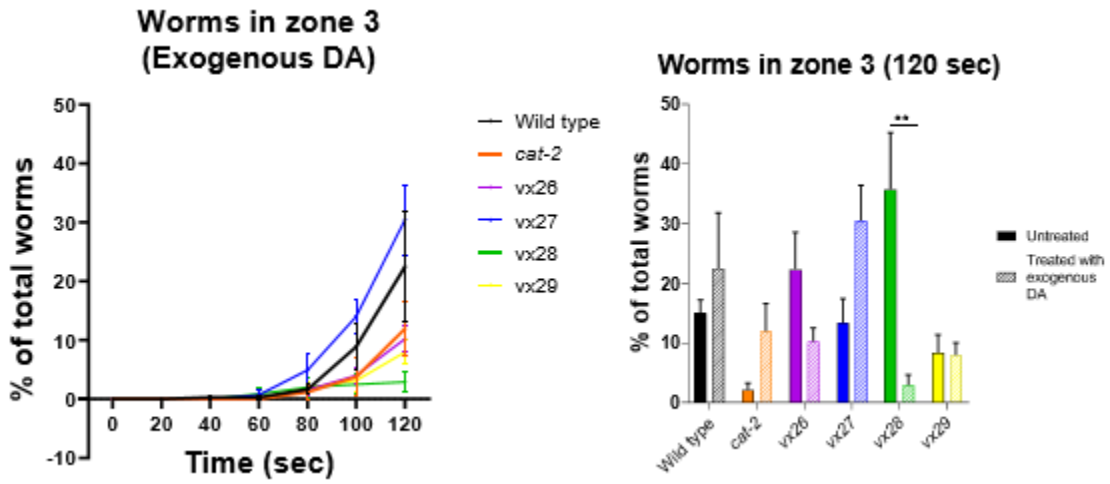

Figure 7: Motor function of suppressor mutants following exposure to exogenous dopamine. Radial dispersion assays were performed on populations of worms exposed to exogenous dopamine. (A) illustrates the proportion of worms that reached the outermost region, zone 3, at each time interval in the 2 minute radial dispersion assay. (B) shows the proportion of worms that reached zone 3 at the 2 minute time point for both untreated worms and worms treated with exogenous dopamine.

5. DISCUSSION

Through the forward genetic screen, four suppressor mutants were successfully isolated and exhibited efficient gait transition. The radial dispersion assays performed with each of the mutant strains showed that all were able to transition between different gaits better than *cat-2* worms, with two strains (*vx26* and *vx28*) exhibiting better motor activity than wildtype worms (Figure 3). Moreover, the mutant strains performed better than the *cat-2* strain only when transitioning between swimming and crawling behaviors in a radial dispersion assay. When evaluated in a dry dispersion assay where the worms crawl without switching to a different gait, the difference in locomotor speed between the mutant strains and the *cat-2* strain was nonsignificant (Figure 4). Therefore, the regain of motor function in the suppressor mutants is specific to gait transition and is not a result of general hyperactivity.

By evaluating the behavioral responses of these mutants under various assays, we aimed to further characterize the mechanism by which they overcame their motor deficit.

There are numerous potential pathways that can be altered to help suppress the Parkinsonian motor phenotype in the mutant strains. Due to the known link between dopamine and gait transition in *C. elegans*, a reasonable hypothesis is that the mechanism of suppression occurs within the dopamine signaling pathway itself. Two types of dopamine receptors in *C. elegans* have been functionally characterized: 1) the D1-like receptor, DOP-1, and 2) the D2-like receptors, DOP-2 and DOP-3 (Suo et. al., 2004). These receptors are G-protein coupled receptors that have opposing effects on the dopamine signaling pathway upon activation, as previously described. DOP-1 knockout mutants are known to exhibit a similar inability to transition between gaits as the *cat-2* strain (Vidal-Gadea & Pierce-Shimomura, 2012). Therefore, mutations that lead to constituently active DOP-1 signaling could overcome the *cat-2* motor deficit.

The behavior of the suppressor mutants when exposed to exogenous dopamine provides insight into whether the dopamine signaling pathway is still active in the mutant strains. Wild type worms with normal dopamine signaling are known to exhibit paralysis upon exposure to higher concentrations of exogenous dopamine (Chase, Pepper, & Koelle, 2004). Thus, if DOP-1 signaling is constituently active in the suppressor mutant strains, we expect them to exhibit a similar paralysis due to dopamine hypersensitivity. Two strains, *vx26* and *vx28*, showed decreased locomotor performance in radial dispersion assays following exposure to exogenous dopamine (Figure 7). Given that both strains exhibit efficient gait transition at levels equal to or higher than wildtype in regular radial dispersion assay (Figure 4), it is likely that they are exhibiting dopamine hypersensitivity due to existing active downstream dopamine signaling. It is important to consider that wild-type worms in this experiment did not exhibit the expected paralysis behavior. This could be a result of the lower number of trials with exogenous dopamine performed on the wildtype worms. Additionally, previous experiments exposed the worms to exogenous dopamine for longer time intervals (20 min) than was used in this assay (10 min) (Chase, Pepper, & Koelle, 2004). Thus, further experiments are needed to validate the observed results from this assay.

Moreover, the response of the suppressor mutants to 6-OHDA treatment can help determine whether dopamine production is still active in the mutant strains. Because dopamine production is already impaired in these suppressor mutants, we hypothesize that inducing degeneration of dopaminergic neurons will not have an effect on the gait transition in these mutant strains. Among the majority of the suppressor mutants, there was no significant difference in motor activity between untreated worms and worms exposed to 6-OHDA. Thus, the results are mostly consistent with our hypothesis, and the induced degeneration of dopamine producing neurons does not appear to impact the strains' abilities to transition between gaits. However, it should be noted that the *vx27* strain does trend towards decreased motor function after treatment with 6-OHDA. This could suggest that some level of dopamine production is still active in this strain and contributes to the suppression of the motor deficit.

To determine the general characteristics of each mutant strain, the results from the procedures can be consolidated and examined as a whole. The *vx26* and *vx28* strains consistently show gait transition efficiency at levels similar to or higher than the wildtype strain. Both strains also display dopamine hypersensitivity in the exogenous dopamine assay

and appear to be unaffected by 6-OHDA treatment. These results hint that the mechanism of suppression for these strains makes the downstream component of DOP-1 signaling constituently active. Such mutations may include mutations that prevent GTP hydrolysis or mutations that increase the conformational flexibility of the GCPR, allowing signal transduction without ligand binding (Stoy & Gurevich, 2015). Additionally, the *vx27* strain displays increased performance in response to exogenous dopamine. One potential explanation for this result is that multiple neuronal pathways aid in regulating gait transition within *vx27*; thus, the addition of exogenous dopamine leads to additive effects of both the dopamine signaling pathway and the other method of suppression, resulting in improved motor function. Finally, the *vx29* strain displays higher motor activity than *cat-2* but does not exhibit dopamine hypersensitivity in response to exogenous dopamine and is not affected by induced dopaminergic neuron degeneration. These results suggest that the mechanism of suppression in *vx29* may be due to a mutation in a pathway not directly related to dopamine signaling.

Though dopamine-independent methods of suppression were not fully explored through the performed experiments, other pathways not directly related to dopamine signaling could cause the observed results. Our group previously demonstrated that the neurotransmitter serotonin also plays a role in gait transition; in *C. elegans*, dopamine appears to initiate crawling while serotonin initiates swimming behaviors (Vidal-Gadea et. al., 2011). A balanced ratio of dopamine and serotonin is thus needed to maintain effective motor transition. In the *cat-2* mutant, the deletion of tyrosine hydroxylase and the subsequent lack of dopamine production impairs the dopamine-serotonin balance, potentially exacerbating the inability to transition between gaits. Then, it is possible that serotonin levels in the suppressor mutants have been altered to restore a balance. Further experiments that examine the levels of serotonin in these suppressor mutant strains can determine whether the dopamine-serotonin interaction in these nematodes has been altered to produce the observed motor behaviors. It is important to consider that like humans, *C. elegans* have hundreds of neuropeptides that could be contributing to the regulation of motor patterns; thus, it is difficult to hypothesize which components can suppress Parkinsonian motor function. Therefore, the unbiased approach of the forward genetic screen enables us to better identify potential mechanisms of recovery without guessing or estimation.

In the future, whole genome sequencing (WGS) of the suppressor mutant strains will be performed to identify the individual mutations responsible for the recovery of motor function. To prepare the suppressor mutants for sequencing, they must be first outcrossed against their parent strain, *cat*-2, to remove the random background mutations that are unrelated to locomotion. Once the strains have been successfully outcrossed to minimize background noise, WGS sequencing data will allow us to identify the molecular pathways that can be altered to alleviate dopamine deficiency related motor symptoms. Determining the location, characteristics, and nature of the suppressor mutations will provide further insight into potential mechanisms that overcome the motor deficits characteristic of late stage Parkinson's disease.

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